



Alternative pathways as mechanism for the negative effects associated with overexpression of superoxide dismutase

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Abstract

One of the most important antioxidant enzymes is superoxide dismutase (SOD), which catalyses the dismutation of superoxide radicals to hydrogen peroxide. The enzyme plays an important role in diseases like trisomy 21 and also in theories of the mechanisms of aging. But instead of being beneficial, intensified oxidative stress is associated with the increased expression of SOD and also studies on bacteria and transgenic animals show that high levels of SOD actually lead to increased lipid peroxidation and hypersensitivity to oxidative stress. Using mathematical models we investigate the question how overexpression of SOD can lead to increased oxidative stress, although it is an antioxidant enzyme. We consider the following possibilities that have been proposed in the literature: (i) Reaction of H_2O_2 with CuZnSOD leading to hydroxyl radical formation. (ii) Superoxide radicals might reduce membrane damage by acting as radical chain breaker. (iii) While detoxifying superoxide radicals SOD cycles between a reduced and oxidized state. At low superoxide levels the intermediates might interact with other redox partners and increase the superoxide reductase (SOR) activity of SOD. This short-circuiting of the SOD cycle could lead to an increased hydrogen peroxide production.

We find that only one of the proposed mechanisms is under certain circumstances able to explain the increased oxidative stress caused by SOD. But furthermore we identified an additional mechanism that is of more general nature and might be a common basis for the experimental findings. We call it the *alternative pathway mechanism*.

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1. Introduction

While most eukaryotes rely on oxygen for their energy production by oxidative phosphorylation, oxygen is usually lethal at concentrations above 40% (Joenje, 1989). It is thought that not oxygen itself but its reactive derivatives like the superoxide radical ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (HO^{\bullet}) and singlet oxygen ($^1\text{O}_2$) are the damaging agents. Oxygen radicals cannot only damage proteins, membranes and DNA (Pryor, 1973; Wolff et al., 1986; Davies, 1987; Mene-

ghini, 1988), but they have also been implicated in the aging process (Harman, 1956, 1981; Fleming et al., 1981). The superoxide radical is produced by several enzymes like xanthine oxidase, indoleamine dioxygenase, tryptophan dioxygenase and aldehyde oxidase (Halliwell and Gutteridge, 1989), but the most important sources of $\text{O}_2^{\bullet-}$ in vivo are the electron transport chain of mitochondria and the endoplasmic reticulum. The generation of superoxide radicals by mitochondria is estimated to be 1–2% of the cellular oxygen uptake (Joenje, 1989; Chance et al., 1979). To counteract the threat posed by oxidative stress cells have developed an impressive arsenal of enzymatic and low molecular weight compounds to remove and detoxify free oxygen radicals. The most important enzymes involved in this

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process are superoxide dismutase (SOD) which catalyses the dismutation of superoxide radicals to hydrogen peroxide (McCord and Fridovich, 1969) and catalase as well as glutathione peroxidase (GPX) which both convert hydrogen peroxide into water. Several forms of SOD exist which can be classified by their metal cofactor. The copper/zinc enzyme (CuZnSOD) is found in the cytosol of eukaryotes, in the chloroplasts of some plants and as a special extracellular version (EC-SOD). Recently, CuZnSOD has also been detected in many bacteria. Manganese SOD (MnSOD) exists in prokaryotes and eukaryotes, but in the latter appears exclusively in the mitochondrial matrix. An iron containing form (FeSOD) is constitutively expressed in prokaryotes and some chloroplasts (for review, see Bannister et al., 1987) and recently a dismutase with nickel as cofactor (NiSOD) has been described in *Streptomyces* and cyanobacteria (Youn et al., 1996; Palenik et al., 2003).

Since SOD catalyses the first step in this chain of important antioxidant reactions it has been tempting to speculate that increasing the concentration of SOD might confer additional resistance to oxidative stress or prolong lifespan. Many studies have been performed to investigate this idea, but surprisingly most of them showed that high levels of SOD have deleterious effects. Groner et al. (1986) could show that transfected cells producing high levels of CuZnSOD suffer from increased lipid peroxidation. Also other cell culture experiments found that overexpression of CuZnSOD leads to hypersensitivity to oxidative stress which could be counterbalanced by increased catalase (Amstad et al., 1991) or GPX levels (Amstad et al., 1994). Using transgenic animals it could be shown that this negative effect is also present at the organismic level. Transgenic *Drosophila melanogaster* with elevated CuZnSOD activity exhibited heavy mortality during development (Orr and Sohal, 1993) and died during the process of eclosion (Reveillaud et al., 1991). Increased lipid peroxidation is also associated with Down syndrome (DS) (Brooksbank and Balazs, 1984) a frequent human genetic disorder with three copies of chromosome 21 (trisomy 21). The gene for CuZnSOD is on chromosome 21 and the increased oxidative stress seen in those patients is thought to be caused by the elevated CuZnSOD activity (Sinet, 1982). Two groups produced mice transgenic for CuZnSOD as an animal model for DS and both found severe impairments like lipid peroxidation, diminished prostaglandin synthesis and serotonin uptake and abnormal neuromuscular junctions (Avraham et al., 1988; Schickler et al., 1989; Minc-Golomb et al., 1991; Ceballos-Picot et al., 1991, 1992). Finally, also bacteria overexpressing FeSOD or MnSOD showed increased sensitivity to paraquat (Bloch and Ausubel, 1986; Scott et al., 1987; Siwecki and Brown, 1990). Some studies (for instance Chan et al., 1998; Keller et al., 1998), did report

beneficial effects of overexpressing SOD. But even in these cases increased oxidative stress is often associated with the elevated SOD levels (Keller et al., 1998).

The most popular interpretation brought forward to explain the observed detrimental effects is that increased levels of SOD directly lead to an increase in the steady-state concentration of hydrogen peroxide, the product of the dismutation reaction (Groner et al., 1986, 1990; Scott et al., 1987; Avraham et al., 1988; Yarom et al., 1988; Kelner and Bagnell, 1990; Seto et al., 1990; Amstad et al., 1991, 1994; Ceballos-Picot et al., 1991; Minc-Golomb et al., 1991; Reveillaud et al., 1991; Mao et al., 1993; de Haan et al., 1995; Zhong et al., 1996; Keller et al., 1998; Schwartz and Coyle, 1998).

However, this simple scenario cannot be correct if we only consider the core reaction of SOD. The important point is that under steady-state conditions the rate of superoxide generation has to be identical to the rate of superoxide removal. That means if we only consider the

simple chain of reactions $\rightarrow \text{O}_2^{\bullet-} \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 \xrightarrow{\text{cat/GPX}} \text{H}_2\text{O}$, it can easily be shown that the amount of SOD controls the steady-state concentration of superoxide, but does not at all influence the hydrogen peroxide steady-state level. The following two equations describe such a system:

$$\begin{aligned} \frac{d\text{O}_2^{\bullet-}}{dt} &= c_1 - c_2 \cdot \text{SOD} \cdot \text{O}_2^{\bullet-} \\ \frac{d\text{H}_2\text{O}_2}{dt} &= c_2 \cdot \text{SOD} \cdot \text{O}_2^{\bullet-} - c_3 \cdot \text{cat} \cdot \text{H}_2\text{O}_2 \end{aligned}$$

Solving this system for steady-state conditions gives us $\text{O}_{2,ss}^{\bullet-} = c_1/c_2 \cdot \text{SOD}$ and $\text{H}_2\text{O}_{2,ss} = c_1/c_3 \cdot \text{cat}$. The hydrogen peroxide level is completely independent of SOD. Only the enzyme that removes H_2O_2 can influence its concentration. This result is independent of the reaction law used. It also holds if we had used a Michaelis Menten kinetics instead of the simpler mass action law or if we had assumed a reversible instead of an irreversible kinetics for the reactions for SOD and cat/GPX.

If high levels of SOD cannot directly lead to increased oxidative stress, how else might the observed negative effects be mediated? Obviously, the simple reaction described above, is not sufficient to explain the observed phenomena. It seems necessary to include additional chemical reactions to understand this counter intuitive behaviour and find explanations how SOD can exert an influence on hydrogen peroxide or other molecular species conferring oxidative stress.

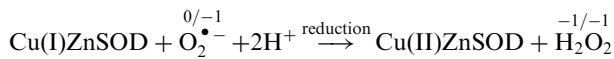
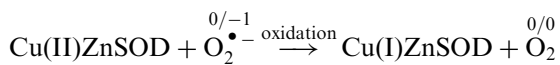
In the present study we therefore investigated three other ideas that have been proposed as possible explanations. We found that only one of these mechanisms operates as expected under certain conditions. However, using model simulations we discovered a new, more general, mechanism that might account for the

observations. We call this the *Alternative Pathway* mechanism.

2. Model description

Three further ideas have been proposed in the literature to explain increased oxidative stress associated with increased levels of SOD. Our intention is to construct a model that is complex enough to represent all these ideas and to formalize this model using differential equations. Computer simulations will then test if the ideas are consistent and show possible interactions. The ideas that are described by the model are as follows:

1. The mechanism of SOD is based on a cyclic process during which the catalytic metal centre is reduced and re-oxidized. Exemplified for CuZnSOD the two reactions of this cycle are as follows (the numbers above the oxygen species indicate the oxidation state):



If an electron donor other than superoxide would reduce the Cu(II) to Cu(I), SOD would act as a superoxide reductase (SOR). And if superoxide would be replaced as electron acceptor in re-oxidizing Cu(I) to Cu(II), the enzyme would act as superoxide oxidase (SOO) (Liochev and Fridovich, 2000; Offer et al., 2000). The important difference is that under normal conditions one molecule of hydrogen peroxide is generated for two molecules of superoxide, while a SOR produces one H₂O₂ for every O₂^{•-} consumed.

2. Superoxide can initiate lipid peroxidation via its conjugated base, the perhydroxyl radical HO₂[•] (superoxide itself is not reactive enough (Halliwell and Gutteridge, 1999)). However, it has also been suggested that superoxide radicals can actually serve as terminator of lipid peroxidation by reacting with lipid peroxyl radicals to yield lipid hydroperoxides: LOO[•] + O₂^{•-} + H⁺ → LOOH + O₂ (Nelson et al., 1994). Over-scavenging of O₂^{•-} by large amounts of SOD could then lead to increased net lipid peroxidation by increasing the chain length.
3. It has been known for many years that in addition to its SOD activity CuZnSOD has also a peroxidase function (Hodgson and Fridovich, 1975). Although the details are still unresolved more recent work indicates that by reacting with H₂O₂ the enzyme can act as free radical generator (Yim et al., 1990, 1993; Goss et al., 1999; Sankarapandi and Zweier, 1999).

This way increased CuZnSOD might lead to increased oxidative stress.

Typically the concentrations of radicals and reactive oxygen species (ROS) are very low (only a few molecules per cell) so that stochastic effects become apparent. If it can be expected that these effects are relevant for the behaviour of the described system, a stochastic modelling approach should be chosen. For instance, this would be the case if signal transduction pathways or the effects of transcription factors are modelled. There it makes a large difference if zero or one molecule is present, since a single copy can serve as trigger to start a complex response. For low levels of radicals, however, stochastic modelling does not seem necessary, since radicals do not act as such triggers (at least not in the reactions included in the model).

2.1. Overview of the model

Fig. 1 gives an overview of the model reactions that incorporate the above points. Labels v_x denote the different reactions and for enzymatically catalysed reactions (v₆ and v₇) the participating enzyme is

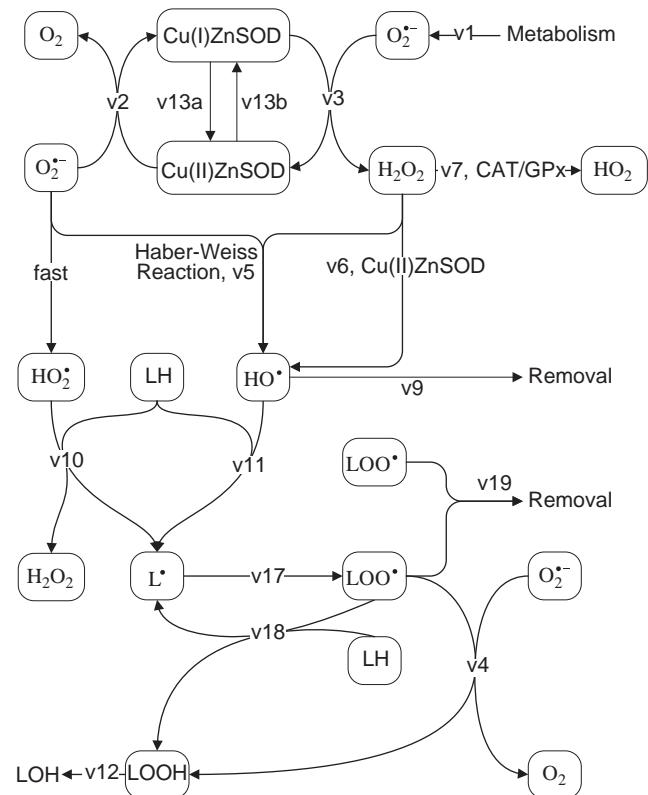


Fig. 1. Reactions included in the model to study three proposals for explaining the negative effects of high levels of superoxide dismutase (SOD). In brief, short circuiting of the SOD cycle is modelled by reactions v₂, v₃, v_{13a} and v_{13b}, termination of lipid peroxidation by reactions v₁₀, v₁₁, v₁₇, v₁₈, v₁₉ and v₄ and peroxidation activity of CuZnSOD by reaction v₆. For details see text.

indicated. While in these reactions the enzymes are unchanged, a special case exists for reactions v_2 and v_3 . These reactions are enzyme catalysed, but the enzyme itself is modified during the reaction and therefore CuZnSOD is shown as substrate and product, respectively. It is assumed that $O_2^{\bullet-}$ is produced by the cellular metabolism at a constant rate (reaction v_1) and can then undergo several different reactions. (i) it can react with Cu(II)ZnSOD and produce O_2 (the SOO part of the dismutation cycle), (ii) it can react with Cu(I)ZnSOD and generate H_2O_2 (the SOR part of the dismutation cycle), (iii) together with H_2O_2 it can give rise to HO^\bullet radicals via the iron catalysed Haber–Weiss reaction, (iv) it can react with lipid peroxy radicals, v_4 , interrupting the lipid peroxidation cycle and (v) it stands in fast equilibrium with its conjugated base the perhydroxyl radical, HO_2^\bullet .

The possibility of short cutting the SOD cycle (proposal one) is realized by the reactions v_{13a} and v_{13b} . If the corresponding rate constants have different values either SOO or SOR activity is favoured.

To test proposal two, reactions describing the lipid peroxidation cycle have to be implemented. Initiation of lipid peroxidation is caused by species that are reactive enough to abstract hydrogen atoms from lipid molecules. In the model HO^\bullet and HO_2^\bullet can start the chain reaction via reactions v_{10} and v_{11} . Under aerobic conditions lipid carbon radicals (L^\bullet) react with oxygen to produce lipid peroxy radical (LOO^\bullet) via reaction v_{17} . Peroxy radicals are capable of abstracting protons from other lipid molecules leading to a lipid hydroperoxide and another L^\bullet (v_{18}). This represents the propagation stage of lipid peroxidation. This chain reaction can be interrupted in two ways. Either two LOO^\bullet molecules can react with each other (v_{19}) in a process of self-termination, or, as proposed, lipid peroxy radicals can interact with superoxide, which now acts as chain breaker (v_4).

It is not completely clear, if the peroxidase activity associated with the reaction between CuZnSOD and hydrogen peroxide is caused by the release of hydroxyl radicals or carbonate radical anions (Yim et al., 1990; Goss et al., 1999). However, in our model the reaction is implemented as possible production of hydroxyl radicals from H_2O_2 (v_6).

Finally, hydrogen peroxide is removed from the system by the enzymatic action of catalase or GPX. For simplicity this is represented as the action of a single enzyme (v_7). Please note, that for clarity species whose concentrations are assumed to be constant (e.g. water, oxygen, protons, metal ions), are sometimes omitted from the diagram. The following list summarizes the reactions included in the model. As explained in the next section, the mass action law has been used for all reactions and the label k_x denotes the rate constants used for the reactions. A list with a description and the

numerical values used for the rate constants is given in Table 1.

- (1) metabolism $\xrightarrow{k_1} O_2^{\bullet-}$
- (2) $O_2^{\bullet-} + \text{Cu(II)ZnSOD} \xrightarrow{k_2} O_2 + \text{Cu(I)ZnSOD}$
- (3) $O_2^{\bullet-} + 2H^+ + \text{Cu(I)ZnSOD} \xrightarrow{k_3} H_2O_2 + \text{Cu(II)ZnSOD}$
- (4) $O_2^{\bullet-} + LOO^\bullet + H^+ \xrightarrow{k_4} O_2 + LOOH$
- (5) $O_2^{\bullet-} + H_2O_2 \xrightarrow{k_5} O_2 + 2HO^\bullet + e^-$
- (6) $H_2O_2 \xrightarrow{k_6, \text{Cu(II)ZnSOD}} 2HO^\bullet$
- (7) $H_2O_2 \xrightarrow{k_7, \text{CAT/GPX}} H_2O + \frac{1}{2}O_2$
- (8) $HO^\bullet \xrightarrow{k_9} \text{metabolism}$
- (9) $HO_2^\bullet + LH \xrightarrow{k_{10}} L^\bullet + H_2O_2$
- (10) $HO^\bullet + LH \xrightarrow{k_{11}} L^\bullet + H_2O$
- (11) $LOOH \xrightarrow{k_{12}} LOH + \frac{1}{2}O_2$
- (12) $\text{Cu(I)ZnSOD} \xrightarrow{k_{13a}} \text{Cu(II)ZnSOD} + e^-$
- (13) $\text{Cu(II)ZnSOD} + e^- \xrightarrow{k_{13b}} \text{Cu(I)ZnSOD}$
- (14) $L^\bullet + O_2 \xrightarrow{k_{17}} LOO^\bullet$
- (15) $LOO^\bullet + LH \xrightarrow{k_{18}} L^\bullet + LOOH$
- (16) $O_2^{\bullet-} + H^+ \xrightleftharpoons{\text{fast}} HO_2^\bullet$
- (17) $LOO^\bullet + LOO^\bullet \xrightarrow{k_{19}} \text{removal}$

2.2. The equations

Based on the diagram shown in Fig. 1 and the list of reactions, the differential equations (ODE) of the model can be developed. The model consists of seven ODEs controlling the time course of ROS ($O_2^{\bullet-}$, H_2O_2 , HO^\bullet), lipid components (L^\bullet , LOO^\bullet , $LOOH$) and the oxidized form of SODs (Cu(II)ZnSOD). Two algebraic equations calculate the concentration of the reduced form of SOD (Cu(I)ZnSOD) and of the perhydroxyl radical.

In all equations we use the simple mass action law since the molecular species under investigation in vivo do not exist in concentrations which would saturate the enzymes. SOD for instance is present in most tissues at concentrations around 10^{-5} M (Tyler, 1975; Fridovich, 1978) compared to the $O_2^{\bullet-}$ steady-state concentration of 10^{-11} – 10^{-12} M (Chance et al., 1979). Although the individual terms of the equations are normally straightforward, there are a few points worth mentioning. In two steps of the lipid peroxidation, substances are involved that do not appear in the equations (lipids (LH) and molecular oxygen). It is assumed that the concentration of these compounds is constant and can thus be included in the value of the rate constant. Furthermore, the superoxide radical is in fast equilibrium with its conjugated base, the perhydroxyl radical ($HO_2^\bullet \rightleftharpoons H^+ + O_2^{\bullet-}$), with a pK_a of approximately 4.8. With a cellular pH of around 6.8 and the

Table 1
Parameters and standard values used for the simulations

Name	Value	Description
k_1	$6.6 \times 10^{-7} \text{ M s}^{-1}$	Rate of cellular superoxide generation. Calculated from Joenje et al. (1985) .
k_2	$1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Rate constant for the superoxide oxidase (SOO) step of the superoxide dismutase reaction. Assumed to be identical to the overall dismutation rate that was taken from (Halliwell and Gutteridge, 1999).
k_3	$1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Rate constant for the superoxide reductase (SOR) step of the superoxide dismutase reaction. Assumed to be identical to the overall dismutation rate that was taken from (Halliwell and Gutteridge, 1999).
k_4	$10^5 \text{ M}^{-1} \text{ s}^{-1}$	Rate controlling the lipid peroxidation termination activity of $\text{O}_2^{\bullet -}$. Free parameter, since no value was available in the literature.
k_5	$2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	Iron catalysed Haber–Weiss reaction (Halliwell and Gutteridge, 1999).
k_6	$1 \text{ M}^{-1} \text{ s}^{-1}$	Approximate upper limit calculated from the information given by Yim et al. (1990) . Ca. $1\text{--}10 \mu\text{M HO}^\bullet$ were generated within 6 min in a solution containing $1.25 \mu\text{M SOD}$ and $30 \text{ mM H}_2\text{O}_2$.
k_7	$3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	Rate constant for catalase. Taken from (Halliwell and Gutteridge, 1999).
k_9	10^6 s^{-1}	Aggregated reaction rate of hydroxyl radicals with substrates outside the current model. A typical rate constant of $10^9 \text{ M}^{-1} \text{ s}^{-1}$ and a substrate concentration of 10^{-3} M was assumed (Halliwell and Gutteridge, 1999).
k_{10}	1000 s^{-1}	Aggregated reaction rate of perhydroxyl radicals with lipids. Calculated from the rate constant of reaction 3 Antunes et al. (1996) and under the assumption that the lipid concentration is constant at 0.5 M (Antunes et al., 1996).
k_{11}	$2.5 \times 10^8 \text{ s}^{-1}$	Aggregated reaction rate of hydroxyl radicals with lipids. Calculated from the rate constant of reaction 1 Antunes et al. (1996) and under the assumption that the lipid concentration is constant at 0.5 M .
k_{12}	0.38 s^{-1}	Removal of lipid hydroperoxides via phospholipid hydroperoxide GPX. The enzyme concentration is assumed to be constant at $3.8 \times 10^{-8} \text{ M}$ (Antunes et al., 1996) and has been aggregated with the reaction constant from reaction 58 Antunes et al. (1996) .
k_{13a}	0.0087 s^{-1}	Aggregated reaction rate of Cu(I)ZnSOD with a hypothetical electron acceptor different from superoxide. The concentration of the electron acceptor was assumed to be $1 \mu\text{M}$ and the rate constant was estimated to be around $8700 \text{ M}^{-1} \text{ s}^{-1}$ from Fig. 1A from Liochev and Fridovich (2000) .
k_{13b}	0.0087 s^{-1}	Aggregated reaction rate of Cu(II)ZnSOD with a hypothetical electron donor different from superoxide. The concentration of the electron donor was assumed to be $1 \mu\text{M}$ and the rate constant was estimated to be around $8700 \text{ M}^{-1} \text{ s}^{-1}$ from Fig. 1A from Liochev and Fridovich (2000) .
k_{17}	$3 \times 10^4 \text{ s}^{-1}$	Aggregated reaction rate of the autoxidation of lipid carbon radicals with molecular oxygen (present at a constant concentration of 10^{-4} M). Data taken from Antunes et al. (1996) .
k_{18}	7 s^{-1}	Aggregated reaction rate of the propagation stage of lipid peroxidation with lipids at a constant concentration of 0.5 M . Reaction 9 Antunes et al. (1996) .
k_{19}	$8.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	Self-termination of lipid peroxy radicals. Reaction 25 Antunes et al. (1996) .
$\text{SOD}_{\text{total}}$	10^{-5} M^{-1}	Total concentration of CuZnSOD (Tyler, 1975; Fridovich, 1978).
cat	10^{-5} M^{-1}	Concentration of catalase. Assumed to be similar to $\text{SOD}_{\text{total}}$.

Henderson–Hasselbalch equation ($\text{pH} = \text{p}K_a + \log_{10}(\text{base/acid})$), it follows that the HO_2^\bullet concentration is ca. 1% of the superoxide concentration ([Halliwell and Gutteridge, 1999; de Grey, 2002](#)). This is expressed by Eq. (9). Another point is the conservation law regarding the amount of SOD. SOD can exist in a reduced or in an oxidized form, but the total amount is fixed in the model (no synthesis or degradation) and equal to $\text{SOD}_{\text{total}}$. Eq. (8) takes this into account by calculating the amount of Cu(I)ZnSOD from the relationship $\text{Cu(I)ZnSOD} + \text{Cu(II)ZnSOD} = \text{SOD}_{\text{total}}$. Finally, the term $k_9 \cdot \text{HO}^\bullet$ in the equation for the hydroxyl radical needs some explanation. The model contains the main source of hydroxyl radicals in the cell while it only covers a single sink, namely the reactions with lipids via v_{11} . To describe the fact that hydroxyl radicals are also removed from the system by reactions outside the scope of the model, the above term has been introduced. It represents the sum of all other sinks for HO^\bullet .

$$\frac{d\text{O}_2^{\bullet -}}{dt} = k_1 - (k_2 \cdot \text{Cu(II)ZnSOD} + k_3 \cdot \text{Cu(I)ZnSOD} + k_4 \cdot \text{LOO}^\bullet + k_5 \cdot \text{H}_2\text{O}_2)\text{O}_2^{\bullet -} - k_{10} \cdot \text{HO}_2^\bullet \quad (1)$$

$$\frac{d\text{H}_2\text{O}_2}{dt} = k_3 \text{O}_2^{\bullet -} \cdot \text{Cu(I)ZnSOD} + k_{10} \cdot \text{HO}_2^\bullet - (k_5 \text{O}_2^{\bullet -} + k_6 \cdot \text{Cu(II)ZnSOD} + k_7 \cdot \text{cat}) \cdot \text{H}_2\text{O}_2 \quad (2)$$

$$\frac{d\text{HO}^\bullet}{dt} = k_5 \text{O}_2^{\bullet -} \cdot \text{H}_2\text{O}_2 + 2k_6 \text{H}_2\text{O}_2 \cdot \text{Cu(II)ZnSOD} - (k_9 + k_{11}) \cdot \text{HO}^\bullet \quad (3)$$

$$\frac{dL^\bullet}{dt} = k_{10} \cdot \text{HO}_2^\bullet + k_{11} \cdot \text{HO}^\bullet + k_{18} \cdot \text{LOO}^\bullet - k_{17} \cdot L^\bullet \quad (4)$$

$$\frac{d\text{LOO}^\bullet}{dt} = k_{17} \cdot \text{L}^\bullet - k_{18} \cdot \text{LOO}^\bullet - k_4 \text{O}_2^{\bullet-} \cdot \text{LOO}^\bullet - 2 k_{19} \cdot \text{LOO}^\bullet \cdot \text{LOO}^\bullet \quad (5)$$

$$\frac{d\text{LOOH}}{dt} = k_{18} \cdot \text{LOO}^\bullet - k_{12} \cdot \text{LOOH} + k_4 \text{O}_2^{\bullet-} \cdot \text{LOO}^\bullet \quad (6)$$

$$\frac{d\text{Cu(II)ZnSOD}}{dt} = (k_3 \text{O}_2^{\bullet-} + k_{13a}) \cdot \text{Cu(I)ZnSOD} - (k_2 \text{O}_2^{\bullet-} + k_{13b}) \cdot \text{Cu(II)ZnSOD} \quad (7)$$

$$\text{Cu(I)ZnSOD} = \text{SOD}_{\text{total}} - \text{Cu(II)ZnSOD} \quad (8)$$

$$\text{HO}_2^\bullet = \text{O}_2^{\bullet-} / 100 \quad (9)$$

3. Results

3.1. Standard simulation

The set of differential equations was solved numerically with *Mathematica*TM. Fig. 2 shows the results of the *standard* simulation, an integration over time, using the parameter values presented in Table 1. After 1000 s all variables have practically reached their steady-state

concentrations. The kinetics of some variables (HO^\bullet , H_2O_2 , $\text{O}_2^{\bullet-}$) is very fast (reaching equilibrium within a few ms), while it is much slower for others (L^\bullet , LOO^\bullet , LOOH). Since the absolute concentrations of the different species vary by several orders of magnitude, different variables are shown on different scales as indicated by the various axes.

3.2. Short circuiting the SOD cycle

It has been proposed that cellular electron donors can short circuit the SOD dismutation cycle. Parameters k_{13a} and k_{13b} were changed to study this idea. Under standard conditions $k_{13a}/k_{13b} = 1$ so that neither SOO nor SOR activities are favoured. To favour the SOR half-cycle the ratio k_{13a}/k_{13b} was reduced to 1/10 and the steady-state values of the variables were calculated for $\text{SOD}_{\text{total}}$ concentrations from 10^{-6} to 10^{-5} M (Fig. 3a). To be sure that the observed effects stem from the manipulation of the dismutation reaction, the lipid peroxidation part was disabled by setting $k_{10} = k_{11} = 0$. As expected the superoxide radical steady-state concentration drops with increasing amounts of total SOD while the hydroxyl radical concentration has a minimum for intermediate SOD levels. For low concentrations of SOD (and thus high superoxide levels), HO^\bullet increases because of the Haber–Weiss reaction and for high levels of SOD the

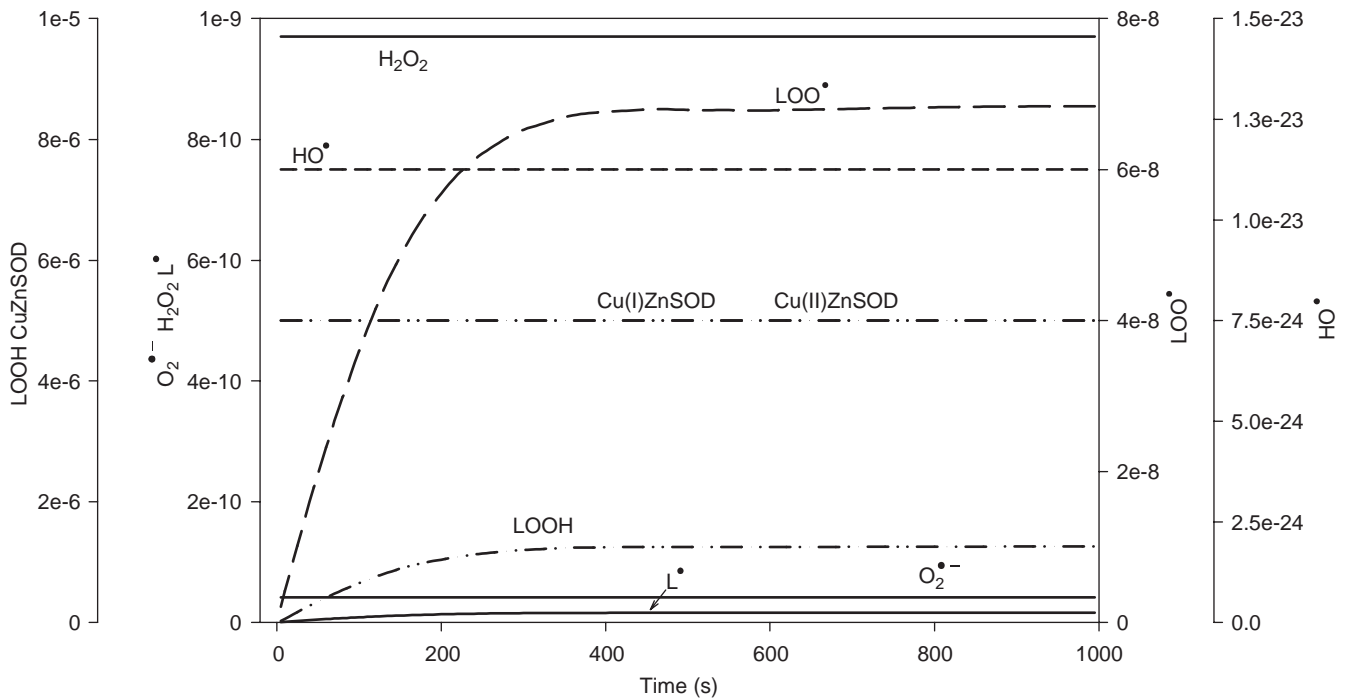


Fig. 2. Time course of the model variables if the simulation is performed with the default parameter values of Table 1. After 1000 s all variables have reached their steady-state concentrations. Because the absolute concentrations of the different variables vary by several orders of magnitude, different scales and axes are used. LOOH and both forms of CuZnSOD are shown on the far left axis, the superoxide radical, hydrogen peroxide and lipid carbon radicals are shown on the left axis, the right axis is for LOO^\bullet and the far right axis shows the concentration of the hydroxyl radical. A similar arrangement is also used for the other diagrams.

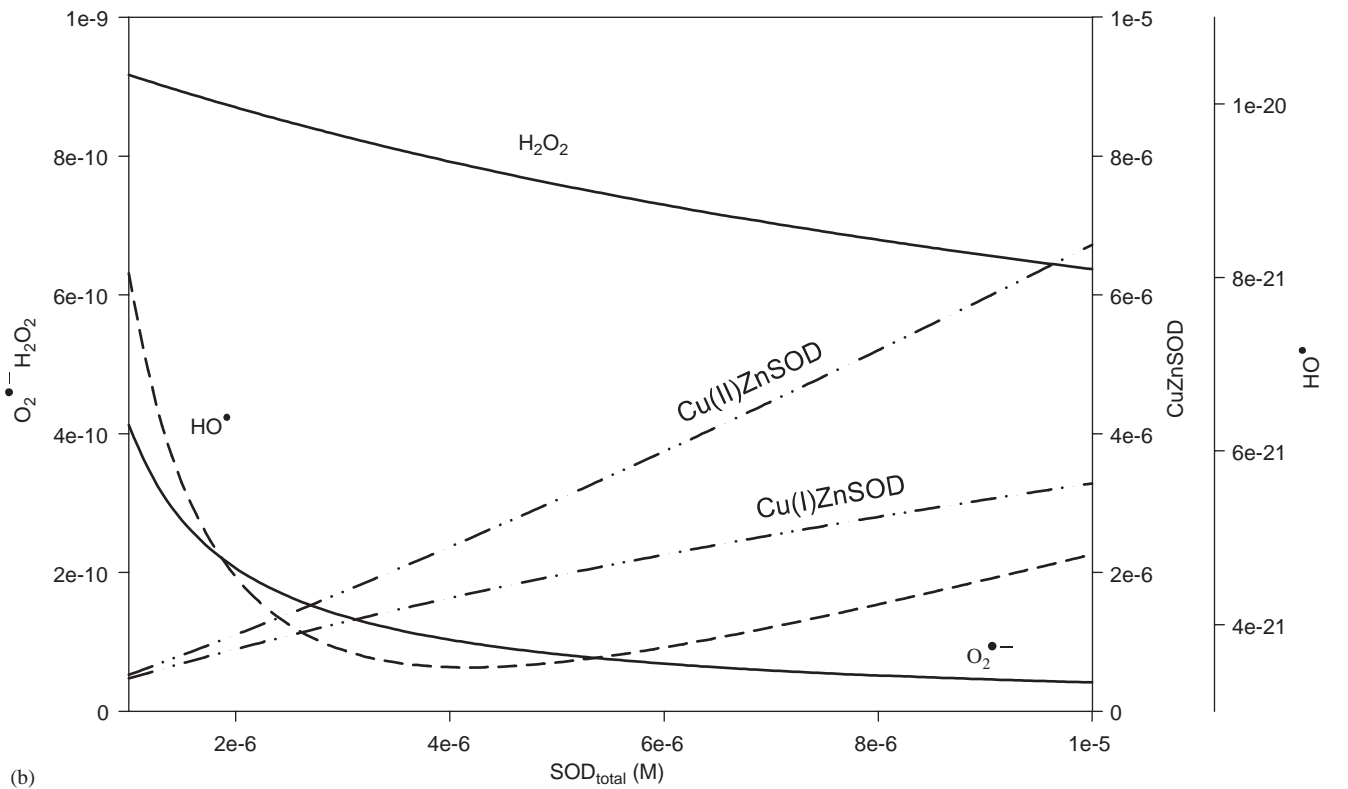
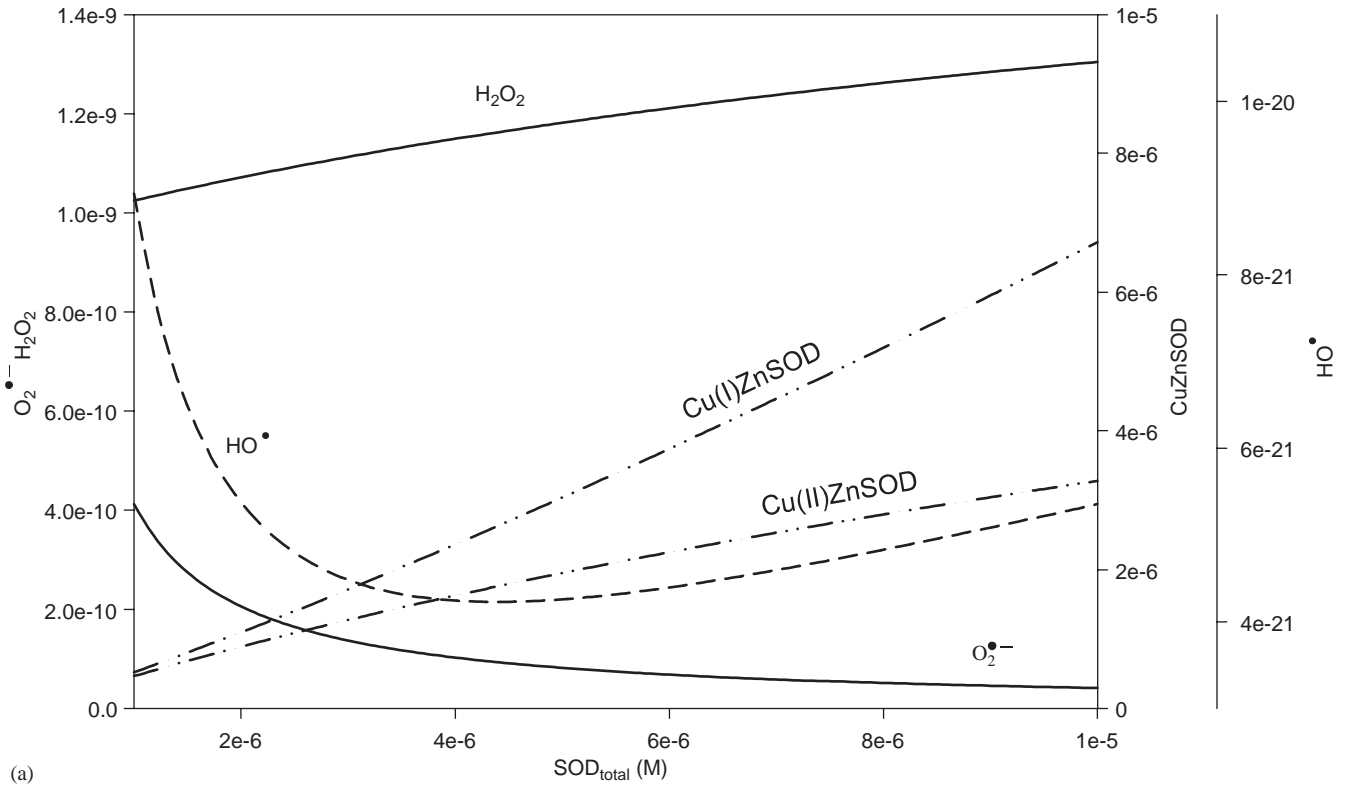


Fig. 3. Effects of short-circuiting the SOD cycle in favour of superoxide reductase (a) or superoxide oxidase (b) activity. To boost SOR activity k_{13b} was set to 10 times k_{13a} and to disable distracting lipid peroxidation reactions k_{10} and k_{11} were set to zero. Under those conditions steady-state values of the shown variables were calculated for SOD_{total} ranging from 10^{-5} to 10^{-7} M. To study the effects of increased SOO activity (b) the same procedure was followed but k_{13a} was set to 10 times k_{13b} .

level of hydroxyl radicals rises because of the SOD peroxidase activity. From Eq. (7) it follows that under steady-state conditions the ratio of reduced to oxidized SOD is given by: $\text{Cu(I)ZnSOD}/\text{Cu(II)ZnSOD} = (k_{13b} + k_2 \cdot \text{O}_2^{\bullet-}) / (k_{13a} + k_3 \cdot \text{O}_2^{\bullet-})$. If total SOD concentration is low and superoxide levels are therefore high the ratio is close to unity (for $k_2 = k_3$). However, if $\text{SOD}_{\text{total}}$ increases and superoxide levels fall, the ratio climbs to k_{13b}/k_{13a} . Consequently this means, that large amounts of $\text{SOD}_{\text{total}}$ favour SOR activity, since more reduced than oxidized SOD is available for the reaction with superoxide radicals. Therefore, the H_2O_2 equilibrium concentration is indeed increasing with the total amount of SOD.

Instead of boosting SOR activity the cell might be in a metabolic state where $k_{13a}/k_{13b} > 1$. While for low SOD concentrations the $\text{Cu(I)ZnSOD}/\text{Cu(II)ZnSOD}$ ratio is still close to one, it decreases to k_{13b}/k_{13a} for high values of SOD. In this case the oxidized form of SOD increases over the reduced form (see Fig. 3b) and consequently the SOO activity is favoured. This leads to a declining H_2O_2 concentration with increasing $\text{SOD}_{\text{total}}$.

3.3. Termination of lipid peroxidation

The toxicity of high doses of SOD led Nelson et al. (1994) to suggest that superoxide radicals can both

initiate and terminate lipid peroxidation. They proposed that $\text{O}_2^{\bullet-}$ can react with lipid peroxy radicals interrupting the peroxidation chain reaction. The rate of this reaction is in our model controlled by the rate constant k_4 . For this reaction no value could be found in the literature and therefore a medium-sized value of $10^5 \text{ M}^{-1} \text{ s}^{-1}$ was used for the standard simulation.

The larger k_4 , the more beneficial should be superoxide for the cell. k_4 was therefore set to a very large value (10^{10}) and the total SOD concentration was varied from 10^{-5} to 10^{-7} M (Fig. 4). However, even with such a large rate constant, high levels of SOD (low levels of superoxide) do not lead to increased steady-state concentrations of L^{\bullet} , LOO^{\bullet} or LOOH . Lipid peroxy radicals seem completely independent of SOD, while L^{\bullet} and LOOH are independent at high levels of SOD and actually increase for low levels. Another important observation is that hydrogen peroxide is actually rising with the amount of SOD.

To understand these simulation results we construct a simpler model that is biochemically less realistic, but can be solved analytically (Fig. 5). In this simplified model superoxide is either removed by SOD, initiates lipid peroxidation (k_2), or terminates it by reaction with LOO^{\bullet} (v_4). This system can be described by the following reactions (constants k_x correspond to the constants of the full model, while constants c_x represent

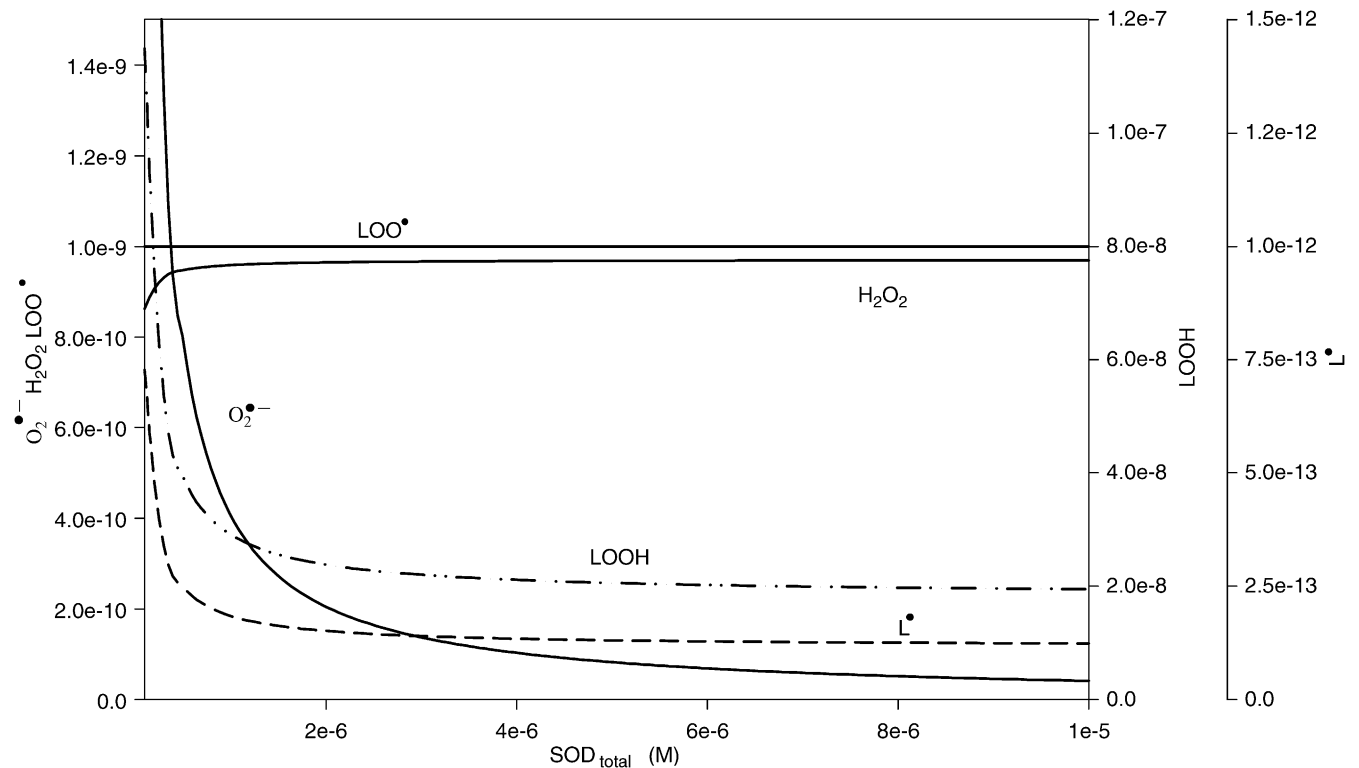


Fig. 4. Influence of SOD on the termination of lipid peroxidation. To increase the relevance of the termination reaction a very large value was used for the rate constant k_4 ($10^{10} \text{ M}^{-1} \text{ s}^{-1}$) and then the steady-state values of the shown variables were calculated for $\text{SOD}_{\text{total}}$ ranging from 10^{-5} to 10^{-7} M . To simplify the interpretation of the results the generation of hydroxyl radicals was disabled for these simulations by setting $k_5 = k_6 = 0$.

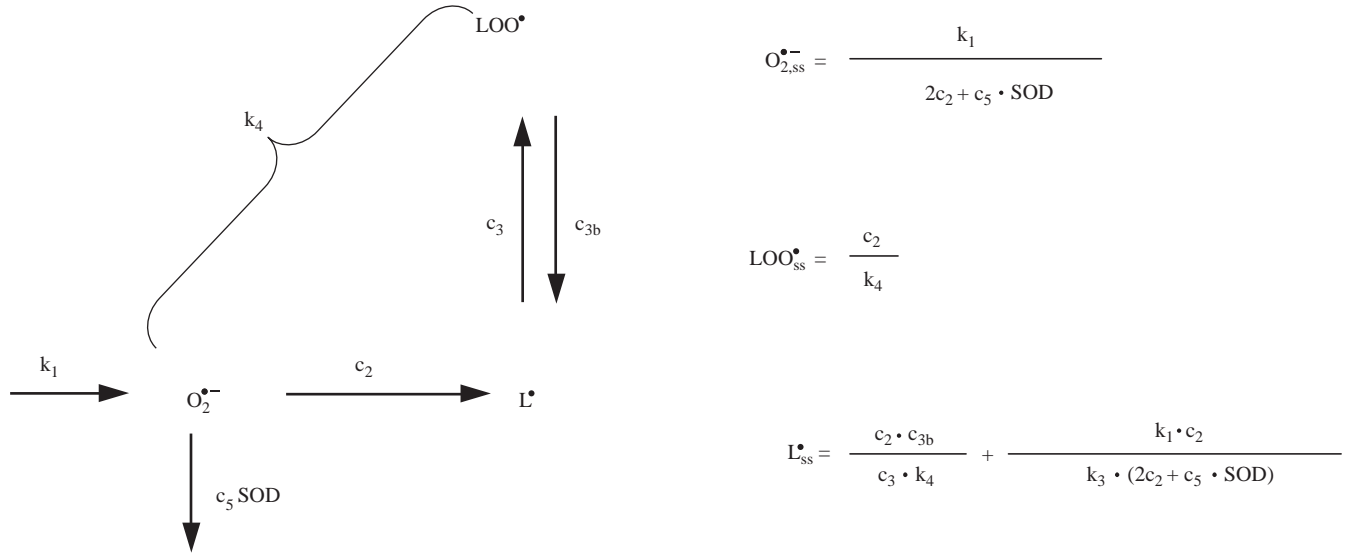


Fig. 5. Simplified model used to understand the behaviour of the model simulation shown in Fig. 4. This system can be described with three simple ODEs (see text for details) that can be analysed analytically. The right part of the diagram shows the dependencies of the steady-state concentrations of the radical species on the parameters. Interestingly, the lipid peroxy radical level does not depend on the amount of SOD.

new constants of this simplified model):

$$\frac{dO_2^{\bullet-}}{dt} = k_1 - (k_4 \cdot LOO^{\bullet} + c_2 + c_5 \cdot SOD) \cdot O_2^{\bullet-}$$

$$\frac{dL^{\bullet}}{dt} = c_2 \cdot O_2^{\bullet-} - c_3 \cdot L^{\bullet}$$

$$\frac{dLOO^{\bullet}}{dt} = c_3 \cdot L^{\bullet} - k_4 \cdot LOO^{\bullet} \cdot O_2^{\bullet-}$$

⇒

$$O_{2,ss}^{\bullet-} = \frac{k_1}{2c_2 + c_5 \cdot SOD}$$

$$LOO_{ss}^{\bullet} = \frac{c_2}{k_4}$$

$$L_{ss}^{\bullet} = \frac{c_2 \cdot c_{3b}}{c_3 \cdot k_4} + \frac{k_1 \cdot c_2}{k_3 \cdot (2c_2 + c_5 \cdot SOD)}$$

The steady-state solutions confirm that lipid peroxy radicals only depend on the ratio of the rates of lipid peroxidation initiation and termination, but not on SOD. L^{\bullet} levels do depend on SOD, but only for low concentrations. Otherwise, the second term tends to zero and the L^{\bullet} concentration is constant and equal to the first term. The simple model predicts that the back reaction of lipid peroxidation (c_{3b}) is responsible for the plateau of L^{\bullet} radicals at high amounts of SOD. Disabling the corresponding reaction in the full model (k_{18}) does indeed cause the lipid carbon radical concentration drop to zero for high SOD levels, verifying the results of the simplified model (data not shown).

The important insight from this analysis is that the equilibrium concentration of LOO^{\bullet} radicals does depend on the rate constant of the proposed termination reaction with superoxide (k_4), but not on the superoxide

concentration itself. The reason is that a change in the $O_2^{\bullet-}$ level affects the initiation of lipid peroxidation to the same degree as the termination. However, these arguments do not explain the rise of hydrogen peroxide. This important phenomenon will be clarified in the section *Alternative Pathway Mechanism*.

3.4. SOD as radical generator

We also studied the effects of the peroxidase function of CuZnSOD. For this purpose we varied the rate constant of this reaction (k_6) from its standard value, $1 \text{ M}^{-1} \text{ s}^{-1}$, to $10 \text{ M}^{-1} \text{ s}^{-1}$ and observed the effects on the steady-state values of the variables. It turns out that the hydroxyl radical level does vary substantially (ca. 9-fold), but the equilibrium concentrations of all other variables changes by less than 0.01% (data not shown). The reason and possible implications of this behaviour are elaborated on in the discussion.

3.5. Alternative pathway mechanism

The simulation shown in Fig. 4 was performed to study the effects of varying SOD concentrations on the reactions involved in lipid peroxidation. A quite unexpected result was the change of the hydrogen peroxide concentration, which was not further explained in the section *Termination of lipid peroxidation*. For the simulation shown in Fig. 4, k_{13a} was equal to k_{13b} so that neither SOO nor SOR activity was favoured and consequently the amount of SOD should have no influence on the H_2O_2 concentration. Closer inspection of this phenomenon revealed that the variation of hydrogen peroxide disappeared when perhydroxyl radical

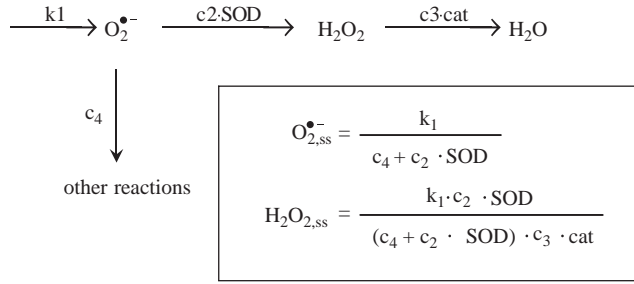


Fig. 6. Simplified model used to understand the *Alternative Pathway* mechanism. This system can be described by two ordinary differential equations (see text for details) that can be solved analytically for the steady-state conditions. The expression for $\text{H}_2\text{O}_{2,ss}$ simplifies to $k_1/(c_3 \cdot \text{cat})$ if there are no other reactions ($c_4 = 0$), making the hydrogen peroxide concentration independent of SOD.

driven membrane damage was disabled ($k_{10} = 0$) (data not shown). To understand this behaviour we use again a simplified version of the full model (Fig. 6). The complete lipid peroxidation part has been omitted and also the rest has been simplified as much as possible. In contrast to the simple reactions discussed in the introduction, the model contains an additional alternative pathway for superoxide radicals (via c_4).

$$\begin{aligned}
 \frac{d\text{O}_2^{\bullet -}}{dt} &= k_1 - c_4 \cdot \text{O}_2^{\bullet -} - c_2 \cdot \text{SOD} \cdot \text{O}_2^{\bullet -} \\
 \frac{d\text{H}_2\text{O}_2}{dt} &= c_2 \cdot \text{SOD} \cdot \text{O}_2^{\bullet -} - c_3 \cdot \text{cat} \cdot \text{H}_2\text{O}_2 \\
 &\Rightarrow \\
 \text{O}_{2,ss}^{\bullet -} &= \frac{k_1}{c_4 + c_2 \cdot \text{SOD}} \\
 \text{H}_2\text{O}_{2,ss} &= \frac{k_1 \cdot c_2 \cdot \text{SOD}}{(c_4 + c_2 \cdot \text{SOD}) \cdot c_3 \cdot \text{cat}}
 \end{aligned}$$

Without alternative pathway ($c_4 = 0$) SOD cancels out of the expression for the H_2O_2 equilibrium concentration. With alternative pathway; however, the dependency remains and rising levels of SOD lead to increased steady-state concentrations of hydrogen peroxide.

4. Discussion

SOD and catalase are two of the major enzymes of the cellular antioxidant system. It has therefore often been proposed that the negative effects seen with high levels of SOD are caused by an increased level of the product of the dismutation reaction, hydrogen peroxide. However, for a simple reaction system consisting only of SOD and catalase or GPX this cannot be an explanation, since under steady-state conditions SOD removes superoxide radicals exactly at the rate of its production. To nevertheless account for the observed increased

oxidative stress associated with elevated levels of SOD, we studied in this work a larger reaction network and investigated three ideas from the literature by developing and simulating a mathematical model of the involved biochemical reactions. This resulted in a system of seven differential and two algebraic equations that were solved numerically using *Mathematica*.

Technically, only one of the three proposals (SOD cycle short circuiting, termination of lipid peroxidation, SOD as radical generator) worked. If an intracellular electron donor exists that short cuts the oxidation/reduction cycle of CuZnSOD by reducing the copper atom at the active site of CuZnSOD from Cu(II) to Cu(I) the enzyme acts predominantly as SOR. Under these conditions an increase in the total SOD concentration leads, as proposed (Liochev and Fridovich, 2000), to an increase of the hydrogen peroxide steady-state levels (Fig. 3a). But there are two points that limit the generality of this mechanism. In their experiments Liochev and Fridovich demonstrated that $\text{K}_4\text{Fe}(\text{CN})_6$ can indeed react as electron donor or acceptor with CuZnSOD. However, they could not show this behaviour with the manganese containing SOD, although increased oxidative stress is also associated with over expression of MnSOD (Bloch and Ausubel, 1986; Omar and McCord, 1990; Zhong et al., 1996). While it can be argued that a suitable redox partner for MnSOD simply has not yet been identified, the more serious problem is that the effect can also work in reverse. If in the cell a substance acts predominantly to oxidize CuZnSOD, and thus enhance its SOO activity, increasing the amount of total SOD actually leads to a reduction of the H_2O_2 level (Fig. 3b). In most experiments performed so far SOD overexpression led to increased oxidative stress, but a priori there is no reason why cells should always be in a state that enhances SOR and not SOO activity. This weakens the generality of the idea.

Termination of lipid peroxidation by superoxide radicals was proposed as beneficial reaction of $\text{O}_2^{\bullet -}$ to explain the negative effects of over-scavenging the radical (Nelson et al., 1994). According to this idea too much SOD would lead to an increased lipid peroxidation chain length and thus to increased oxidative stress. Surprisingly, the simulations show that this idea does not work in our model. The steady-state concentration of lipid radicals and lipid hydroperoxides decreases monotonically with the amount of SOD while lipid peroxy radicals do not depend at all on SOD (Fig. 4). This behaviour finally stems from the fact that superoxide radicals terminate *and* initiate lipid damage so that changes in the radical concentration affect both processes equally. An important message that can be learned from these simulations is that already a small network with few feed backs and non-linearities can display such a complex behaviour that intuition has difficulties to predict the outcome. This demonstrates

how important it is to support verbal ideas by computational simulations.

The third mechanism we explored is the function of CuZnSOD as free radical generator. Simulations showed that the equilibrium concentration of HO^\bullet radicals increases roughly in proportion to rate constant k_6 , but that the levels of the other variables changed by less than 0.01%. The reason is that the hydroxyl radical steady-state concentration is many orders of magnitude below the concentrations of the other molecular species. And although it is extremely reactive, it seems that the main damage to lipids is not caused by the hydroxyl radical, but by the perhydroxyl radical that exists in much higher concentrations. This result is also in agreement with detailed simulations by Antunes et al. (1996).

In addition to the simulation results there are also other arguments that make it unlikely that hydroxyl radicals generated by CuZnSOD or changes of the lipid peroxidation are responsible for the negative consequences of high levels of SOD. In contrast to the other types of SODs MnSOD is resistant to inactivation by H_2O_2 and does not catalyse the formation of hydroxyl radicals (Yim et al., 1990). But at least three reports demonstrated that also overexpression of MnSOD leads to sensitization to oxidative damage (Bloch and Ausubel, 1986; Omar and McCord, 1990; Zhong et al., 1996), which means that in these cases an increased hydroxyl radical production by SOD cannot be responsible for the observed effects. Furthermore, Scott et al. (1987) generated *Escherichia coli* with 10 times increased FeSOD levels and measured directly the amount of hydrogen peroxide produced under oxidative stress. The quantity of H_2O_2 generated after 30 min of 2 mM paraquat treatment was 70% higher in case of the SOD overproducer. So we are looking for a mechanism that influences the hydrogen peroxide level and is not restricted to lipid damage.

In addition to short circuiting the SOD cycle we discovered a further mechanism that influences H_2O_2 levels (Fig. 4), which we termed *Alternative Pathway* mechanism. A mathematical analysis showed that as soon as superoxide radicals have alternative reaction pathways (in addition to the reaction with SOD) increased amounts of SOD do indeed cause increased steady-state levels of H_2O_2 . Although it still holds that under steady-state conditions superoxide is generated at the same rate with which it is removed, we have now the option to influence the relative flux through the different pathways. Increasing SOD redirects $\text{O}_2^{\bullet-}$ away from the alternative pathways towards the reaction with SOD. This result is quite similar to the conclusions obtained by Gardner et al. (2002) who studied a minimalist mathematical model of superoxide generation and consumption. However, their model did not include hydrogen peroxide consuming steps so that they could

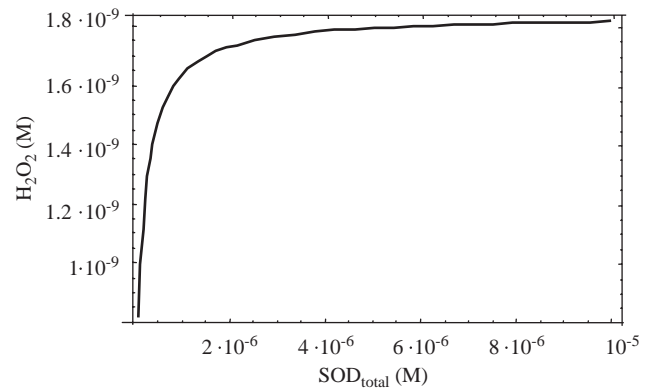


Fig. 7. Effect of the *Alternative Pathway* mechanism on the dependence of hydrogen peroxide on the SOD concentration. This curve was calculated according to the equation shown in Fig. 6. The magnitude of the effect depends strongly on the value of c_4 and for this simulation a c_4 of 20,000, ten times the value used for the other simulations, has been used. Why such a high value might nevertheless be physiological is discussed in the text.

only study production rates and not steady-state levels. They were also not concerned with the evaluation of mechanisms proposed in the literature. The two studies therefore complement and support each other by using different approaches. Another idea along these lines is the work of Buettner et al. (2000). They concentrated on manganese SOD and included variable superoxide production rates in their model. They showed that under certain conditions superoxide removal does actually pull more superoxide into the system, leading to an increase in the flux of H_2O_2 that is directly proportional to the amount of MnSOD.

In the simulation shown in Fig. 4 the hydrogen peroxide concentration did not change much, but in the model only two alternative pathways exist (initiation and termination of lipid peroxidation). In vivo all reactions of superoxide, apart from the reaction with SOD are alternative pathways and thus the flux through these pathways will be much larger than in this model. From the equation for the H_2O_2 steady-state concentration shown in Fig. 6 it can be seen that the hydrogen peroxide level depends linearly on SOD if c_4 tends to infinity. Fig. 7 shows a plot of these equations with a k_{10} value 10 times larger than the standard value used for the simulations. The variation in SOD now leads to a two-fold change in the H_2O_2 equilibrium level.

The alternative pathway mechanism is a very general explanation for SOD associated oxidative stress since it does not depend on a special cell state (SOR activity favoured over SOO activity) nor on specific properties of SOD enzymes (peroxidase activity of CuZnSOD, but not MnSOD). We therefore think that it might be the common mechanism for the detrimental effects seen in cells and organisms with increased levels of the different forms of SOD.

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